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Research Note

Closed-Circuit System for the Depuration of Mussels Experimentally Contaminated with Hepatitis A Virus

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ABSTRACT

In Italy, the consumption of raw or slightly cooked mussels represents the most important risk factor for the transmission of hepatitis A virus (HAV). Although there exist effective methods for the bacterial depuration of contaminated mussels, these methods are poorly effective on enteric viruses. The objective of the present study was to evaluate the effectiveness of a closed-circuit depuration system that uses both ozone and UV light for disinfecting water and that allows salinity and temperature, important parameters for the metabolism of mussels (*Mytilus galloprovincialis*), to be maintained at constant levels. The results showed that this depuration method decreased the viral load (from 1.72 log 50% tissue culture infective dose [$TCID_{50}$] ml^{-1} to <1 log $TCID_{50}$ ml^{-1} within 24 h and from 3.82 log $TCID_{50}$ ml^{-1} to <1 log $TCID_{50}$ ml^{-1} within 48 h). However, in both cases, after 120 h of depuration, a residual amount of virus capable of replicating in cells was detected. These results show that depuration, even if performed with advanced systems, may not guarantee the absence of virus.

In Italy, infection with hepatitis A virus (HAV) is widespread, and large outbreaks have often occurred *(16–19)*. According to the Italian National Epidemiological Surveillance System for Acute Hepatitis Viruses (SEIEVA, Sistema Epidemiologico Integrato per le Epatiti Virali Acute), in the period from 1995 to 1997, cases of HAV infection represented 71% of the notified cases of acute viral hepatitis infection *(5)*.

The most important risk factor for acquiring HAV infection is the consumption of mussels, which has been implicated in 61% of the reported cases in northern and central Italy and in 72% of the cases in southern Italy *(5)*. In fact, molluscs are known to retain bacteria and viruses that are present in the environment, and because they are often eaten raw or only slightly cooked, the risk of illness may be high.

Although the sale of molluscs in Europe is controlled by Directive of Council 91/492/CEE 1991, this law specifies that molluscs can be considered as microbiologically safe for consumption solely on the basis of bacteriological parameters (i.e., *Salmonella* and *Escherichia coli*), yet the absence of bacteria does not necessarily indicate the absence of viruses *(10, 15)*. Moreover, it has been shown that the most commonly used means of performing depuration of molluscs *(26)*, although undoubtedly effective in decreasing the bacterial load, do not guarantee that the viral load will decrease to safe levels *(8, 13)*. To decrease viral load, increasingly sophisticated depuration techniques have been developed; specifically, these techniques include the use of baths of disinfected water in a closed-circuit combined system based on the use of ozone and UV light. They also allow salinity and temperature to be maintained, parameters that affect the filtration process of molluscs *(4, 23)*.

The objective of the present study was to evaluate the effectiveness of this type of depuration process on mussels experimentally contaminated with HAV. To demonstrate the actual elimination of the virus, reverse transcriptase (RT)-nested polymerase chain reaction (PCR) *(9)* to detect viral RNA and integrated procedure cell culture-RT-PCR *(6, 7, 25)* to confirm the presence of infectious virus in the positive samples were used.

MATERIALS AND METHODS

Acclimation and sample contamination. Blue mussels (*Mytilus galloprovincialis*) of medium size (5 to 7 cm in length) from a local seafood market in Rome were used. A cytopathic HAV strain FG *(3, 29)* was grown and titrated in Frp/3 cell culture *(8, 13)*, derived from FrHK/4, both kindly provided by Professor A. Panà of the Università di Tor Vergata, Rome, Italy. Infectivity titer was expressed as 50% tissue culture infectious dose ($TCID_{50}$) ml^{-1} according to the method of Reed and Muench *(28)*. All mussels were acclimatized for 2 days prior to experimental infection in an aerated tank containing 300 liters of artificial seawater (about 1 liter per mussel), prepared using synthetic marine salt (Instant Ocean; Aquarius System, Sarrebourg, France). In the tank seawater the following conditions were maintained: salinity at 30 ± 1‰; water temperature at 15 to 17°C; and the concentration of

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dissolved oxygen in the water at more than 90% of air saturation, as measured using a dissolved oxygen meter YSI 58 (Yellows Springs Inc. Co., Yellow Springs, Ohio). Seawater was contaminated with two different concentrations of HAV (2 log $TCID_{50}$ ml^{-1} or 4 log $TICD_{50}$ ml^{-1} of seawater), and mussels were exposed to the virus for 1.5 h *(13)*.

Mussel depuration. Depuration was conducted in 700-liter commercial baths (length of bath, 160 cm; width, 80 cm; depth of water, 55 cm) with a closed-circuit water-disinfection system that uses both ozone (200 mg h^{-1}) and UV light (λ = 220 to 235 nm), in which a 6-mm layer of seawater passes through a double sleeve that surrounds 110-W lamps 160 cm in length. The seawater was maintained at 30 ± 1‰ salinity and at a constant temperature of 17.8 ± 0.5°C. The water was subjected to a cycle of approximately five complete changes every hour. The mussels were elevated from the bottom of the bath on a perforated plastic tray to curtail recontamination with feces and pseudofeces. Mussels (500 g/sample) were removed at 0, 24, 48, 72, 96, and 120 h for assay. No HAV-contaminated mussels were used as controls.

Each experiment was repeated three times.

Virus extraction. Mussels were rinsed with sterile distilled water: the body and the liquor were removed and homogenized in a blender (Osterizer Pulse Magic 16, Milwaukee, Wis.; 30 s at maximum speed). Seventy-five grams of homogenate was diluted 1:2 in glycine buffer (0.05 M, pH 9.2), stirring for 30 min, and centrifuged at 10,000 × *g* for 15 min at 4°C *(6)*. The supernatant was collected and the pH adjusted to 7.2. Polyethylene glycol (PEG 8,000; Sigma, St. Louis, Mo.) 50% wt/vol solution in 7.5% NaCl (PEG-NaCl solution) at a dilution of 1:4 to obtain a final concentration of PEG 8,000 of 12.5%. The mixture was stirred overnight at 4°C and then centrifuged at 10,000 × *g* for 1 h at 4°C. The pellet was resuspended in 10 ml of 10× Dulbecco's phosphate-buffered saline (Imperial, UK) and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was collected, and PEG-NaCl solution was added at a 1:4 dilution. The mixture was stirred overnight at 4°C and then centrifuged at 10,000 × *g* for 45 min at 4°C. The pellet was resuspended in 3 ml of phosphate-buffered saline and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was extracted twice with 30% chloroform. The interfaces were extracted with 500 μl of cell culture medium (Eagle minimum essential medium with Earle's salts [Imperial]). All the aqueous phases were collected together and centrifuged at 3,000 × *g* for 5 min. To all aqueous phases used for the integrated method, 100× antibiotic-antimycotic (Imperial) solution (1:100 vol/vol) was added and stored at 4°C overnight. The same amount of antibiotic-antimycotic (Imperial) solution was then added, and the sample was maintained at 37°C for 2 h. All the supernatant was stored at −20°C prior to use.

RT-nested-PCR. RNA extraction and purification were performed as previously described *(9)*. In accordance with Afzal and Minor *(1)*, 334 μl of supernatant were added to a 1.5-ml Eppendorf tube containing 666 μl of 1.5 solution D, and the tubes were vortexed for 20 s to 1 min. One hundred microliters of CsCl cushion (5.7 M solution of CsCl in 25 mM sodium acetate pH 5.0) were gently placed at the bottom of the tube by piercing through the liquid. After centrifugation in an Eppendorf microfuge at 13,000 rpm for 20 min at 4°C, the supernatant was discarded, and the pellet was washed twice with 1 ml of 70% ethanol and dried.

RT-PCR. The dried pellet was resuspended in 90 μl of RT reaction mixture containing 1× PCR buffer II (Perkin-Elmer, Branchburg, N.J.), 2.5 mM $MgCl_2$ (Perkin-Elmer), 0.25 mM of each deoxynucleoside triphosphate (Takara-Shuzo, Otsu, Shipa, Japan), 20 U of Rnasin (Promega, Madison, Wis.), 1.25 U of avian myeloblastosis virus RT (Promega), and 100 pmol of primer antisense (5′-CAGGGGCATTTAGGTTT-3′ corresponding to position 669 to 685 HAV strain FG *(3)*); the mixture was incubated at 42°C for 50 min. The reaction was terminated by heating the mixture at 95°C for 3 min. One hundred pmoles of primer sense (5′-CATATGTATGGTATCTCAACAA-3′ HAV strain FG position 1063 to 1084), 2.5 U of *Taq* DNA polymerase (Perkin-Elmer), and DNase-RNase-free water (Sigma) to a final volume of 100 μl were added. The mixture was subjected to 30 PCR cycles, each consisting of 25 s at 95°C, 10 s at 49°C, and 1 min at 70°C. A final extension was carried out for 5 min at 72°C.

Nested PCR. Five microliters of the first amplification reaction were further amplified in 95 μl of reaction mixture containing 1× PCR buffer II (Perkin-Elmer), 2.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 100 pmol of primer antisense (5′-TGATAGGACTGCAGTGACT-3′ HAV strain FG position 807 to 825), 100 pmol of primer sense (5′-CCAATTTTGCAACTTCATG-3′ HAV strain FG position 1,000 to 1,018), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). The amplification conditions used were those described for the first PCR amplification.

Electrophoresis. Ten microliters of PCR and nested-PCR mixture were analyzed by agarose gel electrophoresis (2% agarose; Kodak, New Haven, Conn.).

Integrated cell culture-RT-PCR procedure for qualitative and quantitative detection of infectious HAV. The positive samples were subjected to qualitative and quantitative determination by means of an integrated cell culture-RT-PCR procedure, in order to determine whether the detected viral RNA belonged to infectious viruses, that is, viruses capable of reproducing in the cell cultures *(27)*.

Frp3 cell culture was grown with Eagle minimum essential medium (Imperial) supplemented with 10% fetal bovine serum (Imperial) at 37°C and in 5% CO_2 in 25-cm^2 flasks for 3 days. The cell monolayer was inoculated with 1 ml of mollusc extract and left in contact for 1 h at 37°C and 5% CO_2. The monolayer was then washed three times with 2 ml of Eagle minimum essential medium with 2% fetal bovine serum, in order to eliminate all of the virus not infecting the cells. After adding 5 ml of Eagle minimum essential medium with 2% fetal bovine serum, the monolayer was incubated at 37°C and in 5% CO_2. The cells were observed at regular intervals until the appearance of the cytopathic effect (yet in no case for more than 15 days). Whether the cytopathic effect was present or not, RT-PCR was performed to show the propagation of the virus within the cells.

The samples showing the cytopathic effect in the qualitative determination were subjected to quantitative determination, conducted as previously described *(13)*, using 24-well tissue culture plates and 100 μl of mussel extract. Four replicates were considered for each dilution. The presence of virus in wells with cytopathic effect was confirmed by RT-PCR, carried out as described above using PCR primer.

RESULTS AND DISCUSSION

Tables 1 and 2 show the results regarding the effect of depuration of mussels experimentally contaminated with different concentrations of HAV. The results refer to 1 ml of mussel extract, corresponding to 25 g of mussel homogenate. The mussels immersed in water contaminated with HAV at a concentration of 2 log $TCID_{50}$ ml^{-1} retained,

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TABLE 1. *Effect of depuration on artificially contaminated mussels immersed for 1.5 h in seawater containing 2 log $TCID_{50}$ ml^{-1} HAV*

Time (h)	Qualitative detection: RT-nested-PCR[a]	Integrated procedure[a]: Cell culture[b]	Integrated procedure[a]: RT-PCR	Quantitative detection[a] (log $TCID_{50}$ ml^{-1} ± SD[c])
0	+	+	+	1.72 ± 0.26
24	+	±	+	<1
48	+	±	+	<1
72	+	±	+	<1
96	+	−	+	ND[d]
120	+	−	+	ND

[a] All samples used as control gave negative results.
[b] Cell damage: +, presence of cytopathic effect, disruption of cellular monolayer (death of 90 to 95% of cells) in all determinations; ±, presence of cytopathic effect, disruption of cellular monolayer (death of 90 to 95% of cells) in two out of three determinations; and −, no cell damage.
[c] Mean of three determinations ± standard deviation.
[d] ND, not determined.

TABLE 2. *Effect of depuration on artificially contaminated mussels immersed for 1.5 h in seawater containing 4 log $TCID_{50}$ ml^{-1} HAV*

Time (h)	Qualitative detection: RT-nested-PCR[a]	Integrated procedure[a]: Cell culture[b]	Integrated procedure[a]: RT-PCR	Quantitative detection[a] (log $TCID_{50}$ ml^{-1} ± SD[c])
0	+	+	+	3.82 ± 0.52
24	+	+	+	2.19 ± 0.17
48	+	+	+	<1
72	+	+	+	<1
96	+	−	+	ND[d]
120	+	−	+	ND

[a] All samples used as control gave negative results.
[b] Cell damage: +, presence of cytopathic effect, disruption of cellular monolayer (death of 90 to 95% of cells) in all determinations; and −, no cell damage.
[c] Mean of three determinations ± standard deviation.
[d] ND, not determined.

after 1.5 h, 1.72 ± 0.26 log $TCID_{50}$ ml^{-1} of virus, and those immersed in water contaminated with HAV at a concentration of 4 log $TCID_{50}$ ml^{-1} retained 3.82 ± 0.52 log $TCID_{50}$ ml^{-1} of virus.

All samples subjected to depuration showed a rapid reduction in the quantity of virus retained. In the samples containing HAV 1.72 ± 0.26 log $TCID_{50}$ ml^{-1}, the viral load had decreased to nonquantifiable levels (<1 log $TCID_{50}$ ml^{-1}) already after 24 h. In the samples containing HAV 3.82 ± 0.52 log $TCID_{50}$ ml^{-1}, although there was an evident decrease in the quantity of virus after the first 24 h, only after 48 h did the virus reach nonquantifiable levels. Moreover, regardless of the initial HAV concentration, the virus was shown to be present even after 120 h of depuration. However, after 96 h of depuration, the samples were positive for the presence of viral RNA, but in the qualitative confirmation phase when inoculated on cellular monolayers, the characteristic cytopathic effect was not observed, though the presence of live and infectious virus was demonstrated by the RT-PCR on cellular lysates.

The results obtained indicate that in the first 48 h of depuration, independently of the initial HAV level, the use of this type of bath decreases the viral load to quantitatively undetectable levels. These results differ from those of previous studies that, using the traditional type of bath, revealed the presence of a detectable amount of virus after the same duration of depuration *(8)*. The presence of the virus, even after 120 h of depuration, supports the previous finding that HAV persists in mussel tissue *(12, 24)*. These results show that short-term depuration is not effective in eliminating HAV from mussels. Moreover, such a low quantity of residual virus entails using a sensitive, effective, and rapid method for the direct determination of the virus in mussels, which could be used for routine controls, also because of the inadequacy of the viral contamination index microorganisms (i.e., *E. coli* and phages) *(10)*. However, it should be emphasized that the methods based on the direct use of RT-PCR proposed by various authors *(2, 9, 11, 14)* are rapid and specific *(20)*, yet do not differentiate between infectious and noninfectious viruses and thus require additional confirmation *(15, 27)*. To this end, the integrated method has been shown to be effective, in that its use on positive samples allows only the virus capable of replicating in cells to be detected. Moreover, this method can also be used to detect a virus that is not capable of producing the cytopathic effect, probably because it is present in very low concentrations *(22)*, as in the samples subjected to 96 and 120 h of depuration.

Additional studies should be conducted for developing depuration systems that are more effective in terms of the release of the virus from the body of the mollusc, in order to guarantee its complete elimination. In the meantime, the only sure means of reducing the incidence of viral diseases transmitted through mollusc consumption is to cook molluscs adequately, paying particular attention to the cooking time and temperature *(6, 21)*.

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